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Review

The life cycle of neutral lipids: synthesis, storage and degradation

K. Athenstaedt* and G. Daum

Institute of Biochemistry, Graz University of Technology, Petersgasse 12/2, 8010 Graz (Austria),
Fax: +43 316 873 6952, e-mail: karin.athenstaedt@tugraz.at

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Abstract. Triacylglycerols (TAGs), steryl esters (SEs) and wax esters (WEs) form the group of neutral lipids. Whereas TAGs are present in all types of cell, the occurrence of SEs in prokaryotes is questionable, and the presence of WEs as storage molecules is restricted to plants and a few bacteria. Here, we summarize recent knowledge on the formation, storage and degradation of TAGs and SEs in various cell types. We describe the biochemical pathways involved in TAG and SE synthesis and discuss the subcellular compartmentation of these processes. Recently, several novel enzymes governing the metabolism of storage lipids have been identified and characterized. Regulatory aspects of neutral lipid storage are just beginning to be understood. Finally, we describe consequences of defects in neutral lipid metabolism. Since severe diseases like atherosclerosis, obesity and type 2 diabetes are caused by lipid accumulation, mechanisms underlying neutral lipid synthesis, depot formation and mobilization are of major interest for curing such diseases that are increasingly associated with modern civilization.

Keywords. Triacylglycerol, steryl ester, lipid particle, lipid storage, lipase.

Introduction

The neutral lipids, triacylglycerols (TAGs), steryl esters (SEs) and wax esters (WEs), lack by definition charged groups and are therefore unable to integrate into bilayer membranes in substantial amounts. TAGs and SEs serve as intracellular storage molecules for sterols, free fatty acids and diacylglycerols (DAGs). In algae, plankton and in bacteria, WEs are also used as an energy store [1–4], and in plants and insects they provide the hydrophobic coating of tissues to minimize dehydration of surfaces, and are used for sound transmission and/or buoyancy regulation in sperm whales [1]. Fatty acids stored in neutral lipids can either be used for energy production through β-oxidation or serve as substrates for acylation reactions, e.g. for phospholipid synthesis. DAG released by degradation of TAGs is not only utilized as a substrate for the synthesis of phosphatidylethanolamine and phosphatidylcholine via the Kennedy pathway [5–9], but also acts as a second messenger in signal transduction upon binding to protein kinases [10]. Due to the importance of TAG as an inert storage component, its metabolism appears to be conserved from bacteria to humans. The sterol moieties of SEs are important membrane components which can modulate the physical properties of a bilayer. Furthermore, sterols serve as precursors for the synthesis of steroid hormones and bile acids in higher eukaryotes. Thus, in all types of cell, sterol homeostasis, i.e. the balance between the inactive storage form as SE and the biologically active free sterols, is important. Hydrolysis of WEs liberates fatty acids but can also generate long-chain alcohols which can be further metabolized.

The intracellular storage of neutral lipids occurs in specialized compartments called lipid particles (LPs), lipid droplets or oil bodies. Neutral lipids are sequestered from

* Corresponding author.

the rest of the cell by forming the hydrophobic core of these particles which is surrounded by a phospholipid monolayer with only a few embedded proteins. Upon requirement, neutral lipids are mobilized by the action of lipases and hydrolases, and cleavage products are used as building blocks for membrane formation, as cellular messengers or for energy production (see above).

During the last few years, a number of enzymes involved in the formation, storage and degradation of neutral lipids have been identified at the molecular level. Polypeptides involved in neutral lipid metabolism are well conserved in many species and thus exhibit remarkable homology to each other. There is redundancy for most of the neutral lipid metabolic enzymes. The fact that different subcellular compartments contribute to neutral lipid metabolism in eukaryotes suggests a possible regulation of these processes at the organelle level. These aspects, including consequences of dysfunction in neutral lipid synthesis and/or degradation, are the major subjects of this review article. Since we will focus here on TAGs and SEs, the reader is referred to recent articles about WEs elsewhere [11–13].

Pathways and enzymes catalyzing the formation of TAGs

TAGs are an efficient form to accumulate fatty acids as energy reserves or as building blocks for membrane lipid synthesis. TAGs occur in all eukaryotes (animals, plants, fungi and protists) and also in some prokaryotes, e.g. actinomycetes [14–16], *Streptomyces* species [17] and mycobacteria [18]. The direct precursor for TAG synthesis is DAG, which can be formed by (i) dephosphorylation of *de-novo*-synthesized phosphatidic acid (PtdOH); (ii) degradation of glycerophospholipids through the action of phospholipase C or phospholipase D and phosphatidate phosphatase (PAP); and/or (iii) deacylation of TAGs catalyzed by TAG lipases (Fig. 1).

In prokaryotes, yeast, plants and mammalian cells, PtdOH is synthesized from glycerol-3-phosphate (Gro3P) as precursor. In addition, mammalian and yeast cells can utilize dihydroxyacetone phosphate (GrnP) as a substrate for PtdOH formation. The first step in PtdOH synthesis (see Fig. 1) is the acylation of either Gro3P or GrnP in the sn-1 position leading to 1-acyl-Gro3P (lyso-PtdOH) or 1-acyl-

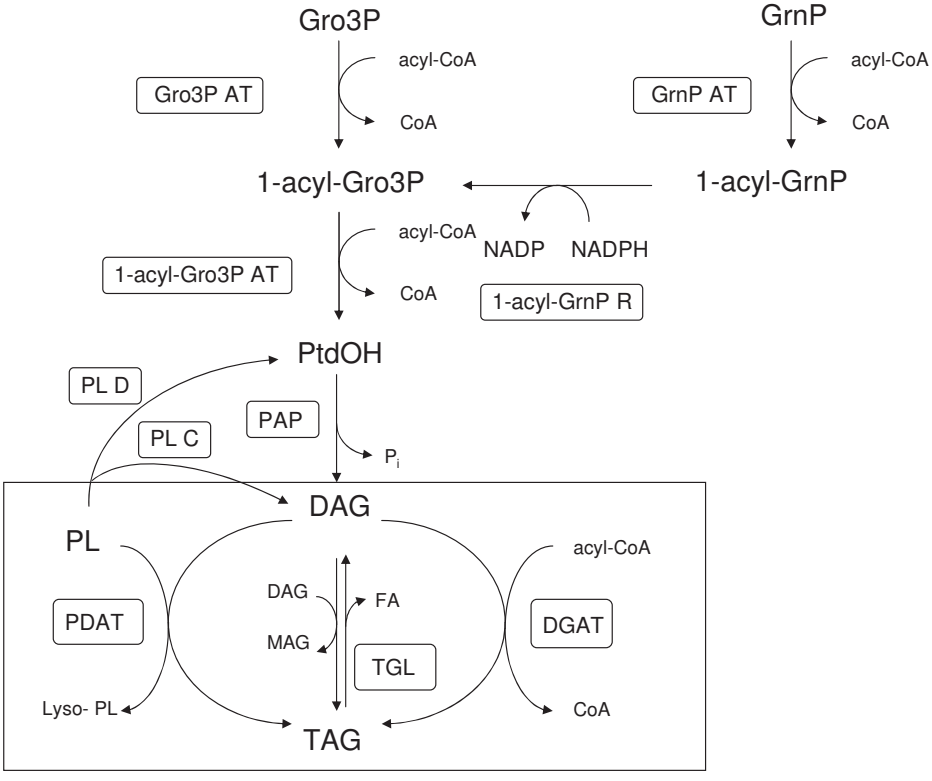

Figure 1. TAG metabolism via different pathways. In the upper part of the figure, enzymatic reactions leading to the synthesis of diacylglycerol (DAG) via the intermediate phosphatidic acid (PtdOH) are shown. The framed, lower part of the figure shows the different possibilities for the final reactions yielding TAG. Enzymes: 1-acyl-GrnP R, 1-acyldihydroxyacetone phosphate reductase; 1-acyl-Gro3P AT, 1-acyl glycerol-3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase; GrnP AT, dihydroxyacetone phosphate acyltransferase; Gro3P AT, glycerol-3-phosphate acyltransferase; PAP, phosphatidate phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PL C, phospholipase C; PL D, phospholipase D; TGL, triacylglycerol lipase. Substrates: 1-acyl-GrnP, 1-acyl dihydroxyacetone phosphate; 1-acyl-Gro3P, 1-acyl glycerol-3-phosphate; DAG: diacylglycerol; FA, fatty acid; GrnP, dihydroxyacetone phosphate; Gro3P, glycerol-3-phosphate; MAG, monoacylglycerol; PL, phospholipid; PtdOH, phosphatidic acid; TAG, triacylglycerol.

GrnP, respectively. Enzymes catalyzing these reactions are Gro3P acyltransferase and GrnP acyltransferase. Work with yeast and mammalian cells showed that at least some enzymes of this type can utilize both substrates [19–21]. In other cases, however, due to lack of information at the molecular level, the question as to the substrate specificity of these enzymes remains open. 1-Acyl-GrnP formed by acylation of GrnP has to be reduced in an NADPH-dependent reaction to yield lyso-PtdOH prior to the second acylation step leading to PtdOH. The second acylation reaction is catalyzed by a 1-acyl-Gro3P acyltransferase.

Prior to the last acylation step leading to the formation of TAG, PtdOH has to be dephosphorylated by a PAP. Esterification of the resulting DAG can be accomplished either by acyl-CoA-dependent or acyl-CoA-independent reactions. In the latter case, enzymes harboring both phospholipase and acyltransferase activities catalyze the transfer of the acyl chain from a glycerophospholipid to DAG. To date, such an enzyme has been identified in yeast at the molecular level and in plants and bacteria by activity [22, 23].

De novo synthesis of TAGs in prokaryotes

In prokaryotes, PtdOH is formed exclusively from Gro3P and subsequently converted to DAG by a PAP. In *Escherichia coli*, only one enzyme exists for each step leading to DAG synthesis [reviewed in ref. 24]. Conversion of DAG to TAG is catalyzed by an acyl-CoA-dependent DAG acyltransferase (DGAT). Since *Mycobacterium* and *Streptomyces* species accumulate large amounts of TAG, DGAT activity of these prokaryotes has been intensively studied [17, 18]. The activity of this enzyme was low during the exponential growth phase, increased upon entry into the stationary phase and decreased again in older cultures [17]. The reduced DGAT activity in stationary cells was paralleled by increased TAG mobilization.

In the Gram-positive bacterium *Rhodococcus opacus* an alternative pathway for TAG synthesis seems to exist because mutants defective in TAG accumulation via the DGAT reaction were demonstrated to be never completely devoid of TAG [23]. An acyltransferase which utilizes phospholipids as acyl donors for TAG synthesis was suggested to catalyze the residual activity in these mutants. Studies with *R. opacus* also demonstrated that the distribution of fatty acid species bound to the glycerol backbone of TAG is non-random. In general, short and saturated fatty acids are found in position sn-2 of TAG, whereas unsaturated fatty acids are preferentially bound to position sn-3. This distribution of fatty acids in bacterial TAG is in contrast to plants and mammals, where long-chain and unsaturated fatty acids are mainly found in the sn-2 position [25]. Cultivation of *Rhodococcus* and *Nocardia* on medium containing pristine, a recalcitrant branched alkane, or phenyldecane as the sole carbon source led to accumulation of TAG containing branched fatty acids and phenyldecanoic acid residues, respectively. Incorporation of unusual fatty acids into bacterial TAG was suggested to prevent their incorporation into membrane lipids where they may disturb properties of the membrane bilayer [for a review see ref. 16].

De novo synthesis of TAGs in eukaryotes

TAG synthesis in yeast

In the yeast *Saccharomyces cerevisiae*, both Gro3P and GrnP can serve as substrates for the synthesis of PtdOH [reviewed in ref. 24]. Localization studies revealed that the entire set of enzymes forming PtdOH via the Gro3P and GrnP pathway are present in LPs and the endoplasmic reticulum (ER). In addition, mitochondria appear to harbor GrnP acyltransferase activity [19]. Dephosphorylation of PtdOH yielding DAG occurs through the action of three PAP isoenzymes [for a review see ref. 26]. One enzyme is bound to the membrane of the ER, one to that of the mitochondria, whereas the third PAP isoenzyme is cytosolic.

As in multicellular eukaryotes, the last step of *de novo* TAG formation in yeast can be accomplished by different routes. In an acyl-CoA-dependent reaction, the activated fatty acid can be linked to the glycerol backbone of DAG by Dga1p [27–29]. Dga1p is a member of the DGAT2 family which comprises a number of DGATs from various species including two LP-located DGATs from the fungus *Mortierella ramanniana* [30, 31]. Enzyme assays using different subcellular fractions from a *dga1* yeast deletion mutant revealed that (i) Dga1p is most likely the only acyl-CoA-dependent DGAT of LP; (ii) Dga1p is also localized to the ER; and (iii) additional enzymes catalyze TAG synthesis in the latter compartment, because TAG synthase activity was only slightly decreased in microsomes of the *dga1* mutant [29]. Enzymatic analysis also revealed that Dga1p has a preference for oleoyl-CoA and palmitoyl-CoA *in vitro* [27].

A second yeast TAG synthase, Lro1p, a homologue of the human lecithin:cholesterol acyltransferase (LCAT), was also identified. In contrast to the human LCAT, Lro1p catalyzes the transfer of an acyl group from the sn-2 position of phosphatidylcholine or phosphatidylethanolamine to DAG [27, 32]. Lro1p activity appears to be restricted to the ER [33]. The relative contributions of Lro1p and Dga1p to TAG synthesis vary with the growth phase. Whereas Lro1p is mainly active during the exponential growth phase, Dga1p exhibits major activity in the stationary phase [27].

In a double mutant deleted for *DGA1* and *LRO1*, however, a minor TAG synthase activity was still detectable [28, 29]. Are1p and Are2p, the two SE synthases of yeast, were shown to be responsible for the residual enzyme ac-

tivity. This observation is in line with the fact that Are1p and Are2p belong to the DGAT1 family which also includes the mammalian DGAT1. Total lack of TAG in a yeast *dga1lro1are1are2* quadruple mutant strongly supported the view of the broad substrate specificity of the Are proteins [28, 34].

In the fission yeast *Schizosaccharomyces pombe,* Dga1p and Plh1p, an acyl-CoA-dependent and acyl-CoA-independent enzyme, respectively, catalyze the final acylation step yielding TAG [35]. A soluble multienzyme complex containing also TAG-synthesizing activity was localized to the cytosol of *Rhodotorula glutinis* [36].

TAG synthesis in plants

Similar to bacteria, plants lack the GrnP pathway and thus synthesize PtdOH only from the precursor Gro3P. In contrast to prokaryotes, however, plants harbor isoenzymes for each acylation step [reviewed in ref. 24]. Soluble acyltransferases have been localized to chloroplasts, and membrane bound enzymes have been detected in microsomes and the outer mitochondrial membrane. There is also redundancy for plant PAPs. Membrane-bound forms have been located to the inner envelope membrane of plastids and to microsomal fractions. Similar to yeast, a cytosolic PAP activity has also been detected in plants [for a review see ref. 24].

The terminal acylation step leading to TAG of plants is catalyzed by a DGAT. This enzyme has broad substrate specificity. Thus, the presence of a particular fatty acid in the sn-3 position of TAG reflects the relative amount of this fatty acid in the acyl-CoA pool [37]. Localization studies with safflower, sunflower, linseed, spinach, avocado and castor bean [38–40] revealed that plant DGATs are microsomal proteins. Plant genes encoding DGAT have been cloned from several species. Comparison of polypeptide sequences revealed that plant DGATs are structurally related to the mammalian DGAT1 [for a review see ref. 41].

In addition to *de novo* synthesis, alternative acyl-CoA-independent routes of TAG formation have been suggested for plants, namely (i) conversion of two DAG molecules to TAG and monoacylglycerol catalyzed by a DAG:DAG transacylase, and (ii) transfer of an acyl moiety from the sn-2 position of a phospholipid to DAG by a phospholipid:DAG acyltransferase [reviewed in ref. 41]. Several candidate phospholipid:DAG acyltransferase genes present in the *Arabidopsis* genome and identified by sequence homology need to be further analyzed for function of the respective gene products.

Plants harbor a wide range of different fatty acids, among them hydroxylated fatty acids, epoxy fatty acids and acetylenic fatty acids. The heterogeneity of the fatty acid pattern is largely confined to TAG from seed lipid depots. Thus, similar to prokaryotic TAG accumulation of unusual fatty acids, TAG may exclude these compounds from plant bilayer membranes to avoid membrane dysfunction. In line with this view, plants storing unusual fatty acids in their seeds were suggested to contain acyltransferases with broad substrate specificity [reviewed in ref. 41].

TAG synthesis in mammalian cells

In mammalian cells, PtdOH can be synthesized either by the Gro3P or the GrnP pathway. There is redundancy for all the enzymes involved. Whereas in the ER both substrates can be acylated, mitochondria utilize only Gro3P and peroxisomes only GrnP as the precursor for TAG formation [21]. Other organelles are devoid of acyltransferase activities [21]. Mammalian enzymes for PtdOH synthesis are localized to different organelles and are differentially regulated [reviewed in ref. 24]. Whereas enzymes located to the ER provide a basic PtdOH pool, PtdOH-synthesizing enzymes of mitochondria and peroxisomes are regulated by hormones and nutrients.

In mammalian cells, two types of PAP are known. One type is localized to the plasma membrane, whereas the other is present in ER and cytosol. Hopewell et al. [42] observed that binding of long-chain fatty acids and their CoA esters to the ER as well as accumulation of PtdOH in this organelle act as signals for enhanced PAP association with the membrane. This association leads to increased synthesis of DAG which is further converted to TAG.

At least two different acyl-CoA-dependent DGATs, namely DGAT1 and DGAT2, catalyze TAG formation in mammalian cells [43, 44]. Surprisingly, these two polypeptides do not share primary structure similarity. The observation that overexpression of several DGAT1 orthologues increased the membrane-associated DGAT activity and the amount of TAG led to the conclusion that expression of DGAT1 is rate limiting in TAG synthesis [43]. Furthermore, treatment of 3T3L1 cells by insulin and dexamethasone led to differentiation of these cells to adipocytes, and increased the amount of DGAT1 mRNA [44]. The concomitant increase in the amount of DGAT1 protein was paralleled by an increase in DGAT activity. Both DGAT1 and DGAT2 were expressed in different mammalian organs at different levels. As an example, a low level of DGAT1 but a high level of DGAT2 expression was found in liver and adipose tissue where TAG synthesis is physiologically important [45, 46]. Most interestingly, Yen et al. [47] reported that DGAT1 not only catalyzes the formation of TAG but also the synthesis of DAG, WEs and retinyl esters *in vitro*. Low DGAT1 expression in liver and adipose tissue may therefore reflect a certain specificity of this enzyme for substrates different from DAG *in vivo*. Since inactivation of DGAT1 does not increase the expression level of DGAT2 in white or brown adipose tissue, expression of the two polypeptides is obviously not coordinately regulated. Mammalian DGAT1 was sug-

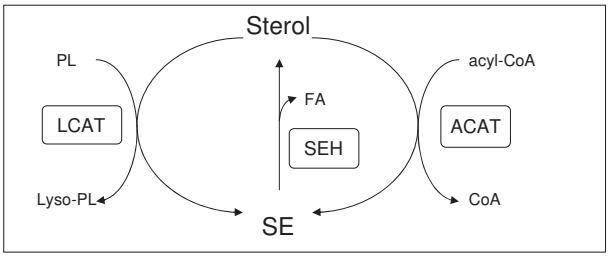

Figure 2. Metabolism of steryl esters. Formation of steryl esters (SEs) is either catalyzed in an acyl-CoA-dependent reaction by acyl-CoA:cholesterol acyltransferase (ACAT) or in an acyl-CoA-independent reaction by lecithin:cholesterol acyltransferase (LCAT). The reverse reaction releasing sterol and a fatty acid (FA) is catalyzed by steryl ester hydrolase (SEH). PL, phospholipids.

gested to be dually located to the ER and LPs like Dga1p of yeast (see above). This finding is consistent with the observation that TAG synthesis in mammalian cells can be accomplished in different subcellular compartments [48]. As in plants, formation of TAGs in mammalian cells can also be accomplished by acyl-CoA-independent reactions. A transacylase activity converting two DAG molecules to TAG and monoacylglycerol was characterized in rat intestinal microsomes [49].

Enzymes catalyzing the formation of SEs

SEs are generally thought to constitute a storage pool of sterols when their cellular amount exceeds the immediate requirement. Synthesis of SEs can be accomplished by two different enzymatic mechanisms (Fig. 2) [43]. In an acyl-CoA-independent reaction, LCAT catalyzes the direct transfer of a fatty acid from a glycerophospholipid to the hydroxyl group in position C3 of the acceptor sterol. This transacylase reaction is similar to that described for TAG synthesis in prokaryotes, yeast and plants (see above). The alternative route of SE formation is acyl-CoA dependent and catalyzed by an acyl-CoA: cholesterol acyltransferase (ACAT). The two routes of SE formation are spatially separated. Whereas synthesis of SE by LCAT occurs in the extracellular space, ACAT esterifies sterols inside the cell.

SE synthesis in prokaryotes and protozoans

The only report about the presence of an SE-synthesizing enzyme in prokaryotes was provided by Thornton et al. [50]. These authors claimed that the bacterium *Aeromonas hydrophila* secreted a phospholipase that exhibited LCAT activity. The secreted enzyme, however, is structurally unrelated to the animal LCAT. Kalscheuer et al. [51] reported that the bifunctional WE/acyl-CoA:diacylglycerol acyltransferase (WS/DGAT) of the Gram-negative bacterium *Acinetobacter calcoaceticus* heterologously expressed in *E. coli* exhibited ACAT activity *in vitro* using cholesterol or ergosterol as substrates. However, heterologous expression of WS/DGAT from *A. calcoaceticus* in a *S. cerevisiae* strain bearing a defect in TAG and SE synthesis restored only the biosynthesis of TAG.

The intracellular protozoan *Toxoplasma gondii* is not able to synthesize sterols by itself and thus has to take up this essential lipid from the host environment. Most recently, Nishikawa et al. [52] identified and characterized two isoforms of ACAT-related enzymes, *Tg*ACAT1α and *Tg*ACAT1β, from *T. gondii*. Both enzymes were located to the ER of the parasite and restored a defect in SE synthesis of the respective mammalian and yeast mutants by heterologous expression. Free fatty acids and low-density lipoproteins not only stimulated the synthesis of SE within the parasite but also led to LP biogenesis. The observation that mRNA levels of *Tg*ACAT1α were significantly increased in fatty acid-stimulated cells suggested a transcriptional regulation of SE synthesis in this parasite.

SE synthesis in yeast

Searches for homologues of the human ACAT [53] led to the identification of two yeast genes, *ARE1* and *ARE2* (ACAT-related enzymes), which encode SE synthases [54, 55]. Analysis of sterol patterns from *are1* or *are2* deletion mutants revealed that Are1p esterifies ergosterol and its precursors with nearly equal efficiency with a slight preference for lanosterol, whereas Are2p uses ergosterol as a preferred substrate [56]. Since ergosterol comprises the majority of yeast cellular sterols, the observed substrate preferences of the two SE synthases may explain the finding that deletion of Are1p had nearly no effect on the total amount of SE whereas a defect in *ARE2* reduced the SE level to approximately 26% of wild type [54, 55]. The total lack of SE in an *are1are2* double-deletion mutant demonstrated that Are1p and Are2p are the only sterol-esterifying enzymes in yeast. Microscopic localization of green fluorescent protein (GFP)-hybrids and enzyme measurements showed that both Are1p and Are2p are localized to the ER [56].

An acyl-CoA-independent pathway for the formation of SEs has not been identified in yeast. The only member of the LCAT gene family in the yeast *S. cerevisiae* is Lro1p, an intracellular protein which, however, catalyzes DAG esterification (see above).

SE synthesis in plants

Plant cells accumulate SE during seed maturation, senescence, at the entrance to the stationary phase and in mutant lines overproducing sterols [57]. Most recently, Banaś et al. [58] identified AtPSAT, the first SE synthase of *Arabidopsis thaliana*. This enzyme was localized to the ER and catalyzes transacylation of acyl groups from the

sn-2 position of phosphatidylethanolamine to sterols. Enzymatic analysis revealed that this enzyme utilizes both saturated and unsaturated fatty acids to esterify sterols and sterol intermediates. Since end products of the sterol biosynthetic pathway activate AtPSAT and the activated enzyme preferentially acylates sterol intermediates, a role for this enzyme in sterol homeostasis was suggested. Because a small but significant amount of SE was still detected in mutants harboring T-DNA insertions in the AtPSAT gene, the presence of additional sterol-acylating enzymes was suggested.

SE synthesis in mammalian cells

Human cells contain two enzymes catalyzing the ACAT reaction. ACAT1 [53], the first enzyme of this kind identified at the molecular level in any organism, and its homologue ACAT2 share 47% amino acid identity. Due to tissue expression patterns and the predicted membrane topology of the two enzymes, ACAT1-derived SEs were suggested to be preferentially incorporated into intracellular LPs, whereas SEs formed through the action of ACAT2 were incorporated into lipoproteins and secreted into the bloodstream [59]. Structural analysis of ACAT1 unveiled two domains near the C terminus of the protein which were critical for catalytic activity. One domain was suggested to mediate binding of the sterol and the other binding of the acyl-CoA group [60]. The hydrophilic N terminus of the polypeptide residing on the cytoplasmic side of the ER is required for tetramerization of the enzyme which negatively influences its enzymatic activity [61].

LCAT of mammals, which is evolutionary unrelated to ACAT, is a soluble enzyme present in the bloodstream. Since LCAT regulates the cholesterol level in the blood, it does not affect the intracellular sterol homeostasis. Interaction of LCAT with apolipoprotein A-1 of high-density lipoproteins (HDLs) was demonstrated by Jonas [62]. Dysfunction of LCAT is associated with classical LCAT deficiency and fish eye disease [63]. An orthologue of LCAT is the LCAT-like lysophospholipase (LLPL) [64] which in contrast to LCAT exhibits phospholipase activity and catalyzes degradation of lysophosphatidylcholine generated by the action of LCAT on HDL particles.

Intracellular storage of neutral lipids

Because neutral lipids such as TAGs and SEs are unable to integrate into phospholipid bilayers, they cluster and form the hydrophobic core of so-called LPs, cell compartments specialized for storage of these compounds. Synonyms for this lipid-storing compartment are lipid droplets, lipid bodies and oil bodies or oleosomes in plants. The structure of LPs is rather simple, resembling that of lipoproteins of mammals [65]. The hydrophobic core of LPs is more or less exclusively formed from TAGs and/or SEs with the ratio of these two lipids depending on the cell type. As an example, in adipocytes, TAG is the main component of LPs, whereas cells specialized for the synthesis of steroid hormones are enriched in SEs. A phospholipid monolayer with a small amount of characteristic proteins surrounds the neutral lipid core of LPs [for a review see ref. 66].

Biogenesis of LPs in prokaryotes

Most recently, Wältermann et al. [67] described a model for LP biogenesis in prokaryotes based on results of *in vivo* and *in vitro* studies. These authors observed that upon docking of WE synthase/acyl-CoA:diacylglycerol acyltransferase to the plasma membrane, small lipid droplets were formed which remained associated with the membrane-bound enzyme. Subsequently, these small lipid droplets clustered, forming membrane-bound lipid pre-bodies which were then released into the cytoplasm. Coalescence of the small lipid droplets inside the lipid pre-bodies led to formation of mature LPs surrounded by a phospholipid monolayer. This model is clearly different from current models of LP formation in eukaryotes (see below).

Biogenesis of LPs in eukaryotes

Despite many efforts, the question as to the origin of LPs in eukaryotes still remains open. Evidence available so far supports the view that LPs originate from the ER. The hypothesis which is currently best accepted for LP biogenesis is a budding model (Fig. 3). According to this model, proteins involved in neutral lipid metabolism accumulate in certain regions of the ER. Among these polypeptides are enzymes catalyzing the formation of TAGs and SEs (Fig. 3a). Because newly formed neutral lipids are unable to integrate into bilayer membranes they cluster and accumulate in the hydrophobic region between the two leaflets of the ER membrane (Fig. 3b). Formation of a neutral lipid droplet between the leaflets may preferentially occur in regions lacking proteins with transmembrane-spanning domains. During ongoing synthesis of TAGs and SEs the droplet grows and forms a bud (Fig. 3c). After reaching a certain size, the LP buds off the ER and is released into the cytosol (Fig. 3d).

Work with the model organism *S. cerevisiae* supported the LP budding hypothesis. First, analysis of the LP proteome revealed that all LP proteins identified so far were involved in lipid metabolism. Secondly, computational analysis of the structure of LP proteins predicted that most, although not all, of these polypeptides lack transmembrane domains [68]. Thirdly, a number of LP

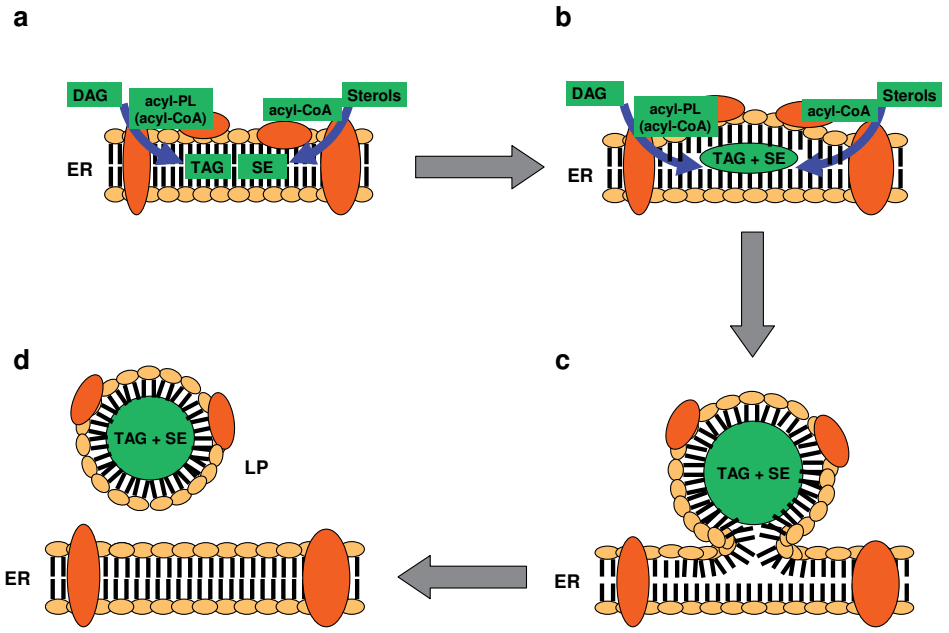

Figure 3. Model for LP biogenesis. (*a*) Synthesis of TAGs and SEs by enzymes of the ER leads to (*b*) the accumulation of TAGs and SEs between the two leaflets of the phospholipid bilayer of the ER. Ongoing synthesis of the neutral lipids results in accumulation of TAGs and SEs, forming a micro-droplet (*c*), which buds off as a mature LP after reaching a certain size (*d*). Acyl-PL, acyl group transferred from a phospholipid to the acceptor DAG. For a detailed description of the budding model of LP biogenesis see the text.

proteins were dually located to LPs and the ER. Finally, in a yeast strain deleted of the genes coding for the TAG- and SE-synthesizing enzymes *DGA1*, *LRO1*, *ARE1* and *ARE2* and consequently lacking LPs, all typical LP proteins were restricted to the ER [34]. Direct evidence for the formation of LPs by a budding process, however, is still missing.

Most recently, Robenek et al. [69] provided evidence for another hypothesis of neutral lipid transfer to the hydrophobic core of LPs. These authors reported that in macrophages and adipocytes, typical LP proteins of the PAT family (perilipin, adipophilin and TIP47) were also integral components of the plasma membrane. Upon stimulation of LP formation by incubating cells with acetylated low-density lipoprotein, proteins of the PAT family clustered in plasma membrane domains, and LPs were closely associated with these areas. Aggregation of the PAT family proteins was suggested to facilitate carrier-mediated lipid influx from the extracellular environment into lipid droplets. LP-specific proteins may be transferred to the particles through interaction with plasma membrane domains enriched in these polypeptides. This process, however, does not describe *de novo* formation but only the increase in size of already existing LPs.

Another way to increase the size of an LP is *de novo* synthesis of neutral lipids by enzymes located on the particle itself. As an example, LPs of the yeast *S. cerevisiae* harbor the entire set of enzymes required for TAG formation. PtdOH formed by LP proteins [for a review see ref. 24] can be converted to DAG by the cytosolic PAP which has access to the surface of LPs. DAG serves as a precursor of the acyl-CoA:diacylglycerol synthase Dga1p, an LP protein [27, 29] forming TAG which can be directly incorporated into the hydrophobic core of the particle. The facts that the activity of enzymes catalyzing PtdOH biosynthesis increases in cells upon entry into the stationary phase [70], and Dga1p contributes predominantly to TAG synthesis in stationary cells [27] are in line with the above-mentioned mechanism. A similar process governing the increase in size of LPs was also predicted for the oleaginous yeast *Y. lipolytica* [71]. Analysis of the LP proteome of *Y. lipolytica* grown either on glucose or transferred to oleic acid-containing medium revealed that, beside the size of the particles, the number of proteins located to this compartment increased upon growth on the latter carbon source. Because these additionally recruited LP proteins completed the set of acyltransferases involved in PtdOH and TAG synthesis on LPs, TAG could be entirely formed by enzymes present on the surface of this compartment.

Beside storage of neutral lipids within the cell in the form of LPs, higher organisms with circulatory systems secrete neutral lipids incorporated into lipoproteins. The structure of lipoproteins [65] is similar to that of intracellular LPs. Secretion of lipoproteins into the circulatory system provides a means to supply cells in the periphery of the organism with energy and metabolites. Neutral lipids

can also be secreted as LPs into milk, allowing the transfer of stored energy from the maternal organism to the offspring. Due to space limitation, however, secretion of neutral lipid-containing particles will not be further discussed in this review article.

Degradation of neutral lipids

When nutrients are no longer provided by the medium, neutral lipids stored in LPs are mobilized by TAG lipases and SE hydrolases. Sterols, DAG and fatty acids set free upon hydrolysis of the storage lipids serve as building blocks for membrane formation, synthesis of steroid hormones and, in the case of free fatty acids and DAG, also for energy consumption. The importance of efficient neutral lipid hydrolysis can be seen from the fact that in humans a number of severe diseases are related to defects in neutral lipid mobilization. As an example, the accumulation of TAG in and the amount of fatty acids released from the adipose tissue have profound implications in the pathogenesis of obesity and diabetes. In mammalian cells and plants, some LP-associated proteins, i.e. the mammalian perilipins and the plant oleosins, have been assumed to act as docking and/or activating proteins for TAG lipases and SE hydrolases. Another function ascribed to perilipins and oleosins is the protection of stored lipids from random degradation [72–76]. Yeast proteins homologous to oleosins or perilipins have not yet been detected [68].

Hydrolysis of TAG in prokaryotes

A number of bacterial TAG lipases have been identified and characterized so far [for recent reviews see refs. 77, 78]. These lipases are an important group of biotechnologically relevant enzymes. Lipases from *Bacillus*, *Pseudomonas*, *Burkholderia* and other prokaryotes are generally produced when bacteria are provided lipidic carbon sources, such as oils, fatty acids, glycerol or Tweens and an organic nitrogen source. Bacterial TAG lipases are serine hydrolases like their eukaryotic counterparts. Most of these lipases are extracellular and can act across a wide range of pH and temperature, although alkaline bacterial lipases are more common. Furthermore, these enzymes exhibit high stability in organic solvents. Some of these lipases are chemo-, regio- and enantioselective, features which have received much attention due to potential applications in biocatalytic processes.

Despite the limited knowledge of neutral lipid metabolism in archaea, enzymes containing TAG lipase activity have been isolated from *Sulfolobus acidocaldarius* [79] and *Thermosyntropha lipolytica* [80].

TAG lipases of yeast

The first TAG lipase identified at the molecular level in the yeast *S. cerevisiae* was Tgl3p [81]. The small but significant residual TAG lipase activity detectable in a *tgl3* deletion mutant indicated, however, the existence of at least one additional TAG lipase. Most recently, two Tgl3p homologues, Tgl4p and Tgl5p, were identified [82]. All three TAG lipases are (i) involved in TAG catabolism *in vivo*, (ii) enzymatically active *in vitro* when purified close to homogeneity, and (iii) localize to LPs. Fatty acid analysis of TAGs from mutants deleted of *TGL3*, *TGL4* and *TGL5* indicated different substrate specificities of the three TAG lipases. Whereas Tgl3p has low substrate specificity regarding the chain length of the fatty acid, TAG of *tgl4* was enriched in myristic acid (C14:0) and palmitic acid (18:0), and TAG of *tgl5* in hexacosanoic acid (C26:0). All three lipases, however, exhibited a high degree of substrate selectivity for TAGs and did not hydrolyze SEs. Although sequence analysis of these three yeast lipases revealed the presence of a GXSXG motif characteristic for lipolytic enzymes, they did not exhibit overall homology to other previously known lipases. Recently, a mammalian homologue of Tgl3p named ATGL [83], and 'Brummer', a homologue in *Drosophila melanogaster* [84], were identified. Most interestingly, heterologous expression of the mouse ATGL reversed only the lipolytic defect of *tgl4* but not of *tgl3* [85]. A lipase of the yeast *Candida parapsilosis* also contains the above-mentioned consensus motif and lacks similarity to other lipases [86].

Degradation of TAG in plants

Lipase activity has been detected at the biochemical level in a variety of plant seeds [87]. In most cases, these activities were only detected upon germination paralleled by a decrease in the amount of TAG. Localization studies revealed the presence of lipases in oil bodies, glyoxysomes and microsomal fractions of seed extracts. However, intracellular translocation of TAG lipases upon specific signals has also to be taken into account. During germination of maize kernel, mobilization of TAGs stored in LPs was shown to occur by a *de novo*-synthesized lipase. Without co- or posttranslational processing, the newly synthesized enzyme bound specifically to LPs [72]. Most likely, oleosins, specific structural proteins of plant LPs, served as docking proteins for the newly synthesized lipase. In addition, oleosins fulfill a structural function, since plant LPs were found to coalesce after treatment with trypsin [88]. It was suggested that during or after lipolysis, the phospholipid layer of the particle fuses with the vacuolar membrane, eventually forming the large central vacuole [89].

Although many lipases from seeds of several plants, e.g. maize, castor and ironweed, have been purified to apparent homogeneity, only recently the first LP-associated

lipase (RcOBL1) from castor bean was identified at the molecular level [90]. Structural analysis of this enzyme revealed the presence of an α/β hydrolase fold, a putative catalytic triad (S-D-H) with the nucleophilic serine residue of the active center present in the conserved GXSXG pentapeptide. Analysis of the substrate selectivity of the castor bean lipase demonstrated that the enzyme hydrolyzed a broad range of different TAG species whereas it was not active against phospholipids. In contrast, lipases purified from seeds of other plants, e.g. oil palm [91], elm [92] and *Vernonia galamensis* [93], revealed high selectivity for specific fatty acids present in TAGs.

Most recently, SDP1 was identified as the first TAG lipase from *A. thaliana* [94]. Most interestingly, this enzyme with its patatin-like acyl hydrolase domain is localized to LPs like its homologues Tgl3p from yeast and ATGL from mammals.

TAG hydrolysis in mammals

In animals, the major energy reserve in the form of neutral lipids is constituted in adipocytes. Mobilization of TAGs stored in adipocytes is tightly regulated by hormones and requires activation of lipolytic enzymes. The first mammalian TAG lipase identified at the molecular level was hormone-sensitive lipase (HSL), a cytosolic enzyme. Upon stimulation, protein kinase A phosphorylates HSL and, simultaneously, perilipin A, a major LP protein which normally protects the neutral lipid core of the particle from degradation. Phosphorylation of both perilipin A and HSL is essential for the translocation of HSL to the surface of LPs where it catalyzes the release of fatty acids from TAG. Analysis of the substrate specificity of HSL revealed, however, that this enzyme not only hydrolyzed TAG but exhibited even higher hydrolytic activity for DAG and cholesterol oleate [95, 96].

For many years, HSL was considered the only mammalian lipase catalyzing degradation of TAG. This view came under scrutiny when residual TAG lipase activity was detected in HSL knock out mice. Indeed, most recently, a novel TAG lipase named adipose triglyceride lipase (ATGL) was identified as an additional TAG lipase [83]. This enzyme is predominantly expressed in white and brown adipose tissue and exhibits high substrate selectivity for TAG, with little or no activity for DAG, SEs, retinyl palmitate or phosphatidylcholine as substrates. Inhibition of ATGL by antisense technology demonstrated that this enzyme was responsible for 70% of the TAG lipase activity in adipose tissue. Since TAG lipase activity was almost completely lost when ATGL was inhibited in an HSL knock out background of white adipose tissue, these two enzymes were suggested to be the major TAG lipases in this tissue. Structural investigations of ATGL revealed that this enzyme contains a patatin domain at the N terminus and the characteristic GXSXG motif similar to the yeast and plant TAG lipases (see above). Moreover, the presence of an α/β hydrolase fold in ATGL was predicted [97].

Lehner and his group cloned TAG lipases from a number of cDNA libraries [reviewed in ref. 98]. These lipases predominantly expressed in liver but also in adipocytes are, however, orthologues of the carboxylesterase multigene family which not only hydrolyze ester but also thioester and amide bonds.

Degradation of TAG in insects

To meet the energy requirements of flight, starvation and egg development, insects utilize stored lipids to survive during physiological non-feeding periods. Lipids and glycogen form the so-called fat body of insects which is functionally comparable to the liver and adipose tissue of vertebrates. Hydrolysis of TAGs from LPs of the insect fat body is (i) catalyzed by a cytosolic TAG lipase [99] and (ii) regulated by the adipokinetic hormone [100]. Stimulation for lipolysis not only leads to phosphorylation of the TAG lipase but also of the LP protein Lsdp1 (lipid storage droplet protein 1) [101], resembling the reaction in mammals (see above). Moreover, another striking analogy between the insect and mammalian system was observed. Similar HSL, phosphorylation of the purified insect lipase did not increase its lipolytic activity. However, use of LPs from stimulated cells as a substrate increased the TAG lipase activity 2.4-fold compared with LPs from non-stimulated cells. Furthermore, treatment of LPs with protein kinase A enhanced phosphorylation of Lsdp1 and subsequently increased the lipolytic activity of the lipase [101].

Most recently, an LP-associated TAG lipase named 'Brummer' was identified in the fruit fly *D. melanogaster* [84]. This lipase is an orthologue of the human ATGL (see above). Chronic overexpression of *bmm*, the gene coding for Brummer, depleted the fat depots of the fly, whereas loss of *bmm* activity caused obesity. Furthermore, fly embryos lacking both maternal or zygotic *bmm* genes were non-viable, demonstrating the essential function of the respective enzyme in *D. melanogaster*. The similarity of TAG lipolysis in mammals and fruit flies may provide the basis for modeling mechanistic and therapeutic aspects of obesity.

Hydrolysis of SEs in yeast

SE hydrolases from the yeast *S. cerevisiae* have evaded molecular characterization for a long time. Only recently, Yeh2p, which is homologous to several known mammalian SE hydrolases, was identified as the first enzyme of this type from yeast at the molecular level [102, 103]. Enzymatic analysis using isolated subcellular fractions and microscopic inspection of a Yeh2p-GFP hybrid demon-

strated that Yeh2p is localized to the plasma membrane. Sterol analysis of *yeh2* unveiled a slight preference of Yeh2p for esters of sterol precursors over esters of ergosterol. Since SE hydrolytic activity was completely lost in the plasma membrane of a *yeh2* deletion mutant, but less affected in other subcellular fractions, the conclusion was drawn that Yeh2p is the only SE hydrolase of the plasma membrane but not in total cell extracts. Indeed, Tgl1p and Yeh1p were identified as additional members of the yeast SE hydrolase family [103, 104]. Localization studies revealed that both Tgl1p and Yeh1p are LP proteins. The complete block in SE hydrolysis in a *tgl1yeh1yeh2* triple-deletion mutant suggested that these three enzymes are the only SE hydrolases of yeast. Since mobilization of TAGs was unaffected in the triple-deletion mutant, these three hydrolases appear to be specific for SEs.

SE hydrolysis in mammalian cells

Mammalian SE hydrolases not only liberate sterols and free fatty acids as building blocks for membrane formation, but also provide large quantities of cholesterol for hormone synthesis in adrenal cells [105] and Leydig tumor cells [106]. Accumulation of SEs in macrophages leads to the formation of foam cells, a critical event in the development of fatty streaks within the arterial wall and progression of atherosclerosis. Therefore, mobilization of SEs is important to keep the level of this lipid low. A number of mammalian SE hydrolytic enzymes have been identified, e.g. carboxyl ester hydrolase, lysosomal acid cholesterol ester (CE) lipase, hepatic cytosolic CE hydrolase, and HSL [for reviews see refs. 107–109]. Enzymatic analysis revealed that these hydrolases not only accept SEs as a substrate but also other molecules such as TAGs. Carboxyl ester hydrolase is mainly synthesized in the pancreas and lactating mammary glands, and secreted. In the extracellular space, it contributes to the digestion and absorption of lipid nutrients. However, this enzyme is also expressed in liver, macrophages and the vessel wall and thus may fulfill multiple functions in lipid and lipoprotein metabolism as well as in atherosclerotic processes [110].

The lysosomal acid CE lipase catalyzes degradation of lipoprotein-associated CE. Cholesterol set free by this reaction can be re-esterified by ACAT1 localized to the ER and deposited in the form of CE in cytosolic LPs [111, 112]. Mobilization of CE stored in LPs is catalyzed by the neutral CE hydrolase (nCEH). Enzymes catalyzing nCEH activity differ among cell types and are distinct from the secreted pancreatic lipase and the acid lipase of the endocytotic pathway. In steroidogenic cells, StAR (steroidogenic acute regulatory protein) interacts with HSL, thereby most likely stimulating the CE hydrolytic activity of HSL. Subsequently, liberated cholesterol is transferred to mitochondria for steroidogenesis [113]. In liver, nCEH may regulate the synthesis of bile acids and thus the removal of cholesterol from the body. This view is in line with recent findings by Zhao et al. [114] who reported identification of two cDNAs from human liver coding for nCEH. These enzymes significantly increased the hydrolysis of CE when heterologously expressed in COS-7 cells and the synthesis of bile acid when overexpressed in human hepatocytes.

The role of nCEH in CE mobilization of macrophages has been studied in some detail because accumulation of CE in these cells leads to the formation of atherosclerotic plaques. Initially, nCEH activity was attributed to HSL because it was also stimulated by cAMP and used TAG and CE as substrates [115]. Since nCEH activity was not reduced in peritoneal macrophages from HSL knockout mice, however, enzyme(s) distinct from HSL were assumed to catalyze this reaction. Indeed, Ghosh and coworkers [116, 117] identified an nCEH distinct from HSL in human macrophages. Variation of the expression level of this protein was paralleled by variation of the amount of stored CE. Most recently, Zhao et al. [118] reported that cytosolic nCEH redistributes from the cytosol to LPs upon lipid loading of human THP-1 macrophages. Depletion of TAG from the particles had no effect on the association of nCEH with this compartment, although the activity of the enzyme decreased when associated with TAG-depleted LPs compared with mixed isotropic droplets.

Defects related to dysfunction of neutral lipid metabolism

In the yeast *S. cerevisiae*, neither the lack of neutral lipid synthesis nor defects in neutral lipid mobilization lead to an obvious growth defect under standard conditions. Nevertheless, neutral lipids appear to be important for some specific processes. As an example, a growth competition experiment showed that a yeast strain deleted of both genes encoding SE synthases, *ARE1* and *ARE2*, exhibited a 65–70% decreased growth rate compared with wild type [56]. Moreover, a mutation of *ARE2* either alone or in combination with *ARE1* affected sporulation efficiency, and a large fraction of sporulating diploids arrested after the first meiotic division [55]. Deletion of *TGL3*, encoding the major yeast TAG lipase, combined deletion of *TGL4* and *TGL5*, and deletion of *TGL1*, encoding one of the yeast SE hydrolases, rendered mutants unable to form spores [82, 104]. In addition, the neutral lipid-free quadruple mutant *dga1lro1are1are2* is impaired in spore formation [82].

The fact that neutral lipid synthesis does not play a major role in budding yeast cultured under laboratory conditions is surprising insofar as TAGs and SEs are important storage forms of biologically active molecules such

as sterols, DAG and fatty acids. Under certain environmental stress conditions, however, the inability to form and store neutral lipids may lead to cytotoxic effects of neutral lipid precursors or intermediates. As an example, the quadruple mutant *dga1lro1are1are2* was reported to undergo programmed cell death (apoptosis) during prolonged growth in the stationary phase and to exhibit a higher sensitivity to fatty acids than the wild type [119]. In contrast to *S. cerevisiae*, mutants of the fission yeast *S. pombe* deleted of genes coding for TAG-synthesizing enzymes lost viability upon entry into stationary phase. These cells showed prominent apoptotic markers including nuclear DNA fragmentation, exposure of phosphatidylserine and generation of reactive oxygen species [reviewed in ref. 119].

Neutral lipids of LPs may also play a role in protein stabilization. As an example, the protein stability of the *S. cerevisiae* squalene epoxidase Erg1p was reduced in an *are1are2* double-mutant strain which lacks SEs. Stability of the Erg1p protein was even more compromised in a *dga1lro1are1are2* quadruple mutant which is totally devoid of neutral lipids [34].

In mammalian cells, dysfunction of neutral lipid metabolism plays a fundamental role in diseases such as atherosclerosis, obesity and diabetes [120–122]. As mentioned above, accumulation of CE in macrophages converts them to foam cells which are involved in the development of fatty streaks within the arterial wall and progression of atherosclerosis. Uptake of CE from lipoproteins into macrophages occurs through the action of specific scavenger receptors, a process which is unregulated. Cholesterol released from lipoprotein-derived CE in a lysosomal process is re-esterified in the absence of an extracellular acceptor and deposited in cytoplasmic LPs. If stored CE exceeds a certain amount, macrophages are converted to foam cells. The balanced synthesis and degradation of CE, known as the 'cholesterol ester cycle', helps to prevent excessive accumulation of CE. For this purpose, either the synthesis of CE has to be inhibited or its hydrolysis has to be stimulated. Inhibition of ACAT activity with progesterone can promote net hydrolysis of CE from lipid-laden mouse peritoneal macrophages [123]. Moreover, net hydrolysis of CE can be increased by the use of an extracellular acceptor for free cholesterol, e.g. HDL, thus stimulating the efflux of free cholesterol [124]. Similarly, overexpression of HSL led to increased net hydrolysis of CE deposited in foam cells [123]. This overexpression alone or in combination with ACAT inhibitors may constitute a useful therapeutic approach for impeding CE accumulation in macrophages *in vivo*.

The lysosomal acid lipase is a key enzyme in the intracellular degradation of neutral lipids which have been internalized through receptor-mediated endocytosis of lipoproteins [125]. Defects in the human lysosomal acid lipase are related to two rare autosomal recessive disorders, Wolman disease and CE storage disease [126]. Wolman disease is lethal within the first year of life due to hepatospenomegaly, adrenal calcification and massive accumulation of TAGs and CE in organs, macrophages and blood vessels. CE storage disease is a less severe disorder exhibiting hepatomegaly, premature atherosclerosis and dyslipoproteinemia. Patients with a milder form of this lipid storage disease retain some residual lipolytic activity [127].

Pancreatic β-cells can store TAGs. The role of these stored lipids, however, is not entirely understood because both beneficial and adverse impacts of the storage of TAGs have been suggested. Requirement of TAG storage for glucose-stimulated insulin secretion was demonstrated by the fact that pancreatic β-cells depleted of this lipid failed to release insulin [128]. In contrast, accumulation of TAG caused β-cell failure and diabetes, a process termed lipotoxicity [129]. Increased formation of TAGs has been shown to exert its toxicity by causing defects in glucose-stimulated insulin secretion [130].

Mechanisms involved in the development of insulin resistance and type 2 diabetes are multifactorial and only partly understood. Elevated concentrations of circulating free fatty acids in the plasma have been suggested to cause the inability of glucose uptake in muscle and liver and thus insulin resistance, hyperinsulinemia, hyperglycemia and dyslipidemia [131–133]. Since hydrolysis of TAGs critically affects the concentration of circulating free fatty acids, inhibition of lipases could provide a means for the treatment of insulin resistance in type 2 diabetes. Specifically, the development of inhibitors for ATGL and HSL, the mammalian TAG lipases, offers novel possibilities for treatment of insulin resistance.

Conclusions and perspectives

Many players in the neutral lipid storage/mobilization game have been identified during the last few years. Some enzymes involved in TAG and SE metabolism have been studied at the functional level, others investigated at the molecular level or even characterized by structure. However, we are only beginning to understand the cell biological role of these enzymes and their products, the neutral lipids. First attempts to ascribe a role in cell development to this class of lipids have been made. Another aspect that is far from being understood is the occurrence and function of neutral lipid-synthesizing and -metabolizing enzymes with overlapping functions. We can only speculate at present that products formed or substrates degraded by these enzymes might be involved in regulatory processes or in spatially limited arrangements of specific lipids. A future focus of research in this area will be to identify additional TAG and SE synthases or hydrolases with minor activity, because such enzymes may be important for

providing and maintaining local cellular pools of certain neutral lipid species. Finally, interorganelle communication appears to play an important role during neutral lipid homeostasis, either by differential targeting of enzymes involved in these processes or by migration of lipid intermediates or products. In summary, although much progress has been made in the field of neutral lipid research, more detailed molecular biological, cell biological and biochemical studies will be needed for a better understanding of the role of neutral lipids in the different kingdoms of life.

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